Chimpanzee/human mAbs to vaccinia virus B5 protein neutralize vaccinia and smallpox viruses and protect mice against vaccinia virus

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Contributed by Robert Purcell, December 9, 2005

Chimpanzee Fabs against the B5 envelope glycoprotein of vaccinia virus were isolated and converted into complete mAbs with human γ1 heavy chain constant regions. The two mAbs (8AH8AL and 8AH7AL) displayed high binding affinities to B5 (K_d of 0.2 and 0.7 nM). The mAb 8AH8AL inhibited the spread of vaccinia virus as well as variola virus (the causative agent of smallpox) *in vitro*, protected mice from subsequent intranasal challenge with virulent vaccinia virus, protected mice when administered 2 days after challenge, and provided significantly greater protection than that afforded by a previously isolated rat anti-B5 mAb (19C2) or by vaccinia immune globulin. The mAb bound to a conformational epitope between amino acids 20 and 130 of B5. These chimpanzee/human anti-B5 mAbs may be useful in the prevention and treatment of vaccinia virus-induced complications of vaccination against smallpox and may also be effective in the immunoprophylaxis and immunotherapy of smallpox.

biodefense

Concerns that variola (smallpox) virus might be used as a biological weapon have led to the recommendation of widespread vaccination with vaccinia virus (VACV) (1). Although vaccination is generally safe and effective for prevention of smallpox, it is well documented that various adverse reactions in individuals have been caused by vaccination with existing licensed vaccines (2). Vaccinia immune globulin (VIG) prepared from vaccinated humans has historically been used to treat adverse reactions arising from VACV immunization (3–6), and, to date, VIG is still the only recommended treatment (5, 6). However, VIG lots may have different potencies and carry the potential to transmit other viral agents.

VACV is the prototype virus in the genus *Orthopoxvirus*, which includes variola virus, the causative agent of smallpox. There are two major forms of infectious VACV: intracellular mature virus (MV) and extracellular enveloped virus (EV). The majority of the MV remains within the cell until lysis, but some are wrapped in additional membranes and exocytosed as EV. Most EV remains attached to the outside of the plasma membrane and is responsible for direct cell-to-cell spread; however, some are released into the medium and can cause comet-like satellite plaques (7, 8). The EV is important for virus dissemination *in vivo* as well as in cultured cells (9, 10). Because an EV is essentially an MV enclosed by an additional membrane, the two forms of VACV have different outer proteins and bind to cells differently (11), although ultimately only the proteins of the MV membrane mediate membrane fusion (12). B5 is one of five known EV-specific proteins and is highly conserved among different strains of VACV as well as in other orthopoxviruses (13, 14). B5 is a 42-kDa glycosylated type I membrane protein with a large ectodomain composed of four small domains that are similar to short consensus repeat (SCR) domains of complement regulatory protein (13, 14), although no complement regulatory activity has been demonstrated. B5 is required for efficient envelopment of MV, as well as for actin tail formation, normal plaque size, and virulence (15–17).

The B5 protein is an important target for neutralizing antibodies: antisera to B5 can neutralize EV in a plaque reduction assay and inhibit "comet formation," the *in vitro* manifestation of cell-to-cell spread of EV (13, 18–20). Recent studies showed that anti-B5 in VIG was responsible for most of the neutralizing activity against EV as measured by a plaque reduction assay (21). To date, rat and mouse anti-B5 neutralizing mAbs have been reported (20, 22), and the epitopes recognized by mouse mAbs have been mapped to the border of SCR1–SCR2 and/or the stalk of B5 (20). In addition, a rat mAb to B5 provided protection in a VACV mouse challenge model (23).

We decided to obtain therapeutically useful high-affinity mAbs to B5 protein from chimpanzees because of the extreme similarity of their IgG with human IgG (24, 25). A phage display library bearing Fabs was derived from the bone marrow of chimpanzees that had been vaccinated with VACV. From this library, we isolated and characterized two potent anti-B5 antibodies that neutralize variola virus in addition to VACV. Such human-like mAbs against B5, in principle, could provide superior protection with a lower dose and higher safety profile than VIG.

Results

Isolation and Characterization of Vaccinia B5-Specific Fabs. The chimpanzee Fab-displaying phage library was panned against recombinant VACV B5 protein (275t), and 96 individual clones were randomly picked and screened for binding to B5 by phage ELISA with BSA as a negative control. Ninety percent of the clones preferentially bound to B5. DNA sequencing of the variable regions of heavy (VH) and light (VL) chains from 18 positive clones showed that a single VH gene was paired with two different VL genes. These two clones were designated 8AH8AL and 8AH7AL (GenBank accession nos. DQ316791, DQ316789, DQ316792, and DQ316790). The sequences of VH and VL genes are shown in Fig. 1 *a* and *b*. A search in V-Base (26) indicated that the VH gene

Conflict of interest statement: No conflicts declared.

Abbreviations: VACV, vaccinia virus; VIG, vaccinia immune globulin; MV, mature virus; EV, enveloped virus; pfu, plaque-forming unit; HRP, horseradish peroxidase; SCR, short consensus repeat; WR, Western Reserve.

Data deposition: The sequences reported in this paper have been deposited in the GenBank database (accession nos. DQ316789–DQ316792).

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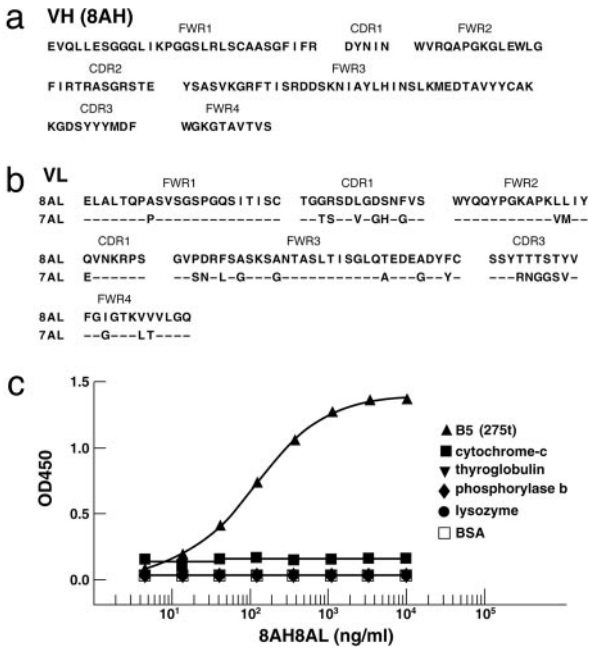

Fig. 1. Amino acid sequences of variable domains of heavy (*a*) and light (*b*) chains of chimpanzee/human anti-B5 mAbs and ELISA titration of anti-B5 8AH8AL (*c*). Complementarity-determining regions (CDR1, CDR2, and CDR3) and framework regions (FWR1, FWR2, and FWR3) are indicated above the sequence or sequence alignment. Dashes indicate an identical residue. For the ELISA binding assay, the wells of ELISA plates were coated with recombinant B5 (275t) or unrelated proteins (BSA, thyroglobulin, lysozyme, and phosphorylase-b) and then incubated with 8AH8AL at various concentrations. Bound IgG was detected by the addition of peroxidase-conjugated anti-human $(Fab)_2$ followed by tetramethylbenzidine substrate.

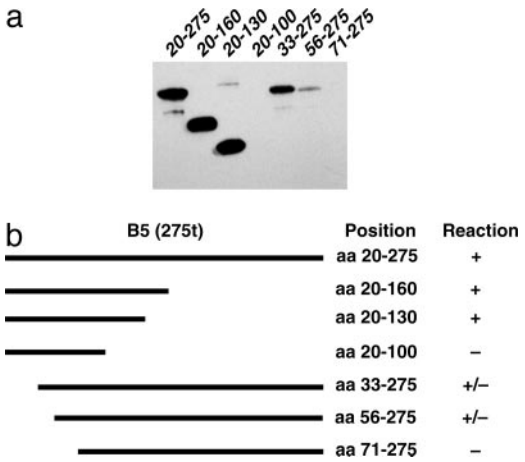

Fig. 2. Epitope mapping by Western blotting. (*a*) Similar amounts of different-sized fragments of B5 expressed in bacteria were blotted onto the membrane, and anti-B5 mAb was added. The bound anti-B5 was detected by HRP-conjugated anti-human IgG $(Fab')_2$. The positive bands were visualized with addition of LumiGLO chemiluminescent peroxidase substrate and exposing the membrane to x-ray film. The result is summarized in *b*, where the peptides that reacted with antibody are scored as positive (+). Faint intensity of the bands is scored as +/−. The numbers denote the starting and ending amino acids.

putatively originated from germ-line gene V3–49, which belongs to the VH3 family; the two VL genes were from V_λ germ-line gene 2a2.272A12, which belongs to the V_λ 2 family.

The Fab sequences were converted into full-length IgG as described in *Methods*, and the IgGs were examined for their binding specificity by ELISA. Anti-B5 8AH8AL bound to B5 protein with high specificity and affinity, but not to unrelated proteins (BSA, thyroglobulin, phosphorylase b, lysozyme, and cytochrome *c*) (Fig. 1*c*). The two anti-B5 mAbs had the identical binding specificity (data not shown).

Epitope Recognized by the Anti-B5 mAb. In the absence of differences in heavy chain sequence, 8AH8AL was chosen for epitope mapping because it had a slightly higher affinity. Western blotting was used to locate the epitope recognized by anti-B5 8AH8AL. Different B5 fragments generated by N- and C-terminal deletions were produced in bacteria. Western blotting with anti-His confirmed that similar amounts of each peptide were tested for reaction with anti-B5 8AH8AL (data not shown). As seen in Fig. 2*a* and summarized in Fig. 2*b*, the shortest peptide that strongly reacted with anti-B5 8AH8AL consisted of residues 20–130 on B5 protein. Therefore, it required 110 amino acid residues to form the epitope, which suggested the epitope was conformational, because a linear epitope is usually composed of 5–15 amino acid residues.

To address whether mAbs can react with its smallpox B5 counterpart, the $B5_{20–130}$ protein of VACV was converted to that of variola virus by means of splicing by overlap extension PCR (see *Supporting Text*, which is published as supporting information on the PNAS web site). Vaccinia and variola virus $B5_{20–130}$ proteins were quantified by Western blotting by using horseradish peroxidase (HRP)-conjugated anti-His (data not shown). The Western blotting with 8AH8AL showed that anti-B5 mAb crossreacted with smallpox $B5_{20–130}$, although the reaction was not as strong as with VACV $B5_{20–130}$ (data not shown).

Binding Affinity and *in Vitro* Neutralizing Activity. The affinity of the two chimpanzee/human mAbs and a rat mAb (19C2) for binding to VACV B5 protein was measured by surface plasmon resonance biosensor. A K_d of 0.6 nM and a dissociation rate constant of $\approx 10^{-5}$ per sec was observed for 8AH8AL (Table 1). A similar K_d was determined in the surface plasmon resonance solution competition assay, both for 8AH8AL (0.2 nM) and for 8AH7AL (0.7 nM). In contrast, the affinity of the rat mAb 19C2 was ≈13-fold weaker (Table 1). Remarkably, the off-rate of the mAb 8AH8AL was 25-fold slower than that of the rat mAb 19C2. Thus, the half-life of the antibody–antigen complex was ≈19 h for the chimpanzee/human mAbs and <45 min for the rat mAb.

Because B5 is an EV-specific protein, *in vitro* neutralization activity of anti-B5 mAbs was measured by the comet-reduction assay, an established method that measures the inhibition of comet-like plaque formation by the released EV form of the virus (27, 28). The EV of the IHD strain of VACV formed comet-shaped plaques in the absence of antibodies, but the formation of comets was completely blocked by the addition of an excess of rabbit hyperimmune serum to VACV (Fig. 3*a*). The monoclonal anti-B5 clones, 8AH8AL and 8AH7AL, reduced the formation of comet-like plaques of VACV EV at the lowest dose tested (Fig. 3*a*). Similarly, the formation of comet-shaped plaques of the Solaimen

Table 1. Antibody affinity of anti-B5 mAbs

Antibody	k_{on}, $M^{-1}s^{-1}$	k_{off}, s^{-1}	K_d, nM
8AH8AL	2×10^4	1×10^{-5}	0.6
19C2	3×10^4	2.6×10^{-4}	7.5

Anti-B5 IgG of chimpanzee/human mAb 8AH8AL and a rat MAb 19C2 were immobilized individually on the surface plasmon resonance sensor surfaces. The antibody binding responses to B5 (275t) protein were collected at a range of concentrations between 0.05 and 500 nM antigen. The kinetic rate constant of association (k_{on}) and dissociation (k_{off}) rates were measured from surface binding kinetics, and the equilibrium dissociation constant (K_d) was calculated as the ratio k_{off}/k_{on}.

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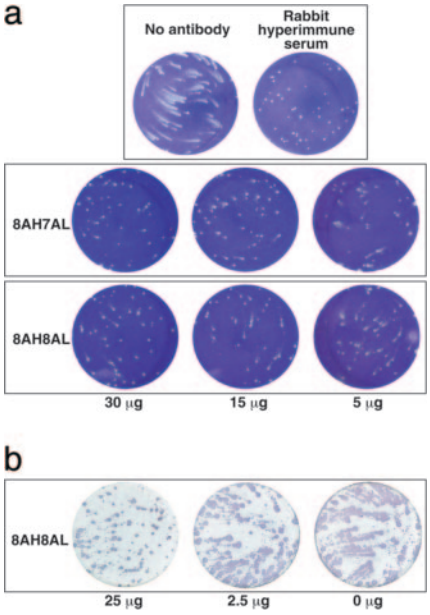

Fig. 3. *In vitro* neutralizing activity of anti-B5 mAbs measured by a comet-reduction assay. (*a*) BS-C-1 cells were infected with ≈50 pfu of VACV, strain IHD-J. After 2 h at 37°C, the monolayer was washed, and fresh medium containing indicated amounts of chimpanzee anti-B5 8AH7AL or 8AH8AL was added. PBS and rabbit hyperimmune serum served as negative and positive controls, respectively. After 48 h, the monolayers were stained with crystal violet. For the smallpox assay (*b*), monolayers of BS-C-40 cells in six-well cell culture plates were infected with the Solaimen strain of variola virus at 50 pfu per well in RPMI medium containing 2% FBS. After 1 h, the medium was aspirated; cells were washed twice and overlaid with RPMI medium containing 25, 2.5, or 0 μg of anti-B5 IgG. The plates were then incubated in a CO_2 incubator for 4 days at 35.5°C. Cells were fixed and reacted with polyclonal rabbit anti-variola virus antibody. After incubation with goat anti-rabbit–HRP conjugate, comets were visualized by addition of TrueBlue peroxidase substrate.

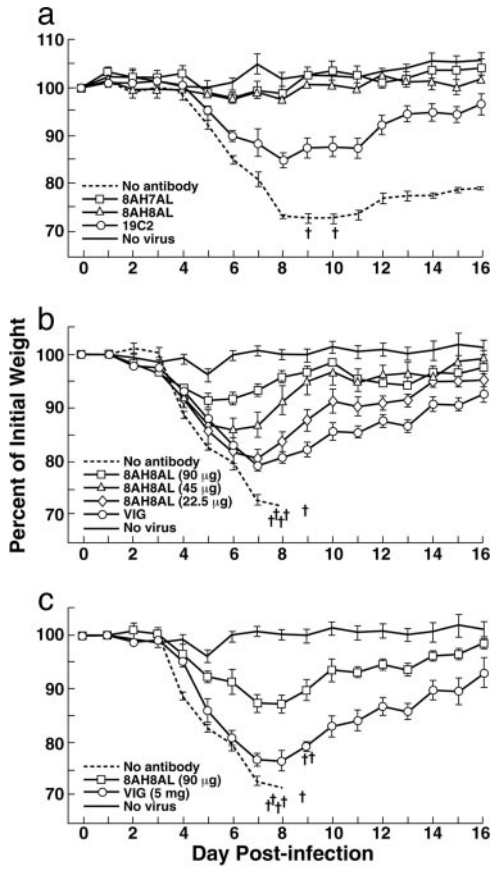

Fig. 4. Prophylactic and therapeutic protection in mice by anti-B5 mAbs. Groups of five BALB/c mice were inoculated i.p. with 90 μg of purified IgG (*a*) or different amounts of IgG (*b*). Twenty-four hours later, mice were challenged intranasally with 10^5 pfu of the WR strain of VACV. Ninety micrograms of rat anti-B5 19C2 IgG (*a*) or 5 mg of human VIG (*b*) were used for comparison. (*c*) Groups of five BALB/c mice were inoculated intranasally with 10^5 pfu of VACV, strain WR. After 48 h, the mice were injected i.p. with 90 μg of purified IgG or 5 mg of human VIG. Mice were weighed individually, and mean percentages of starting weight ± standard error were plotted. Controls were unimmunized (No antibody) or unchallenged (No virus). †, died naturally or were killed because of 30% weight loss.

strain of variola EV was inhibited by 8AH8AL in a dose-dependent manner (Fig. 3*b*), indicating that the anti-B5 mAbs possessed neutralizing activity against EV of both viruses.

Protection of Mice Against Challenge with Virulent VACV. The BALB/c mouse pneumonia model with VACV Western Reserve (WR) challenge (29, 30) was used for the following reasons: weight loss and death are correlated with replication in the lungs, allowing the onset and progress of disease to be monitored by a noninvasive method that reduces the number of animals needed for significance (31); the model has been used for active immunization studies with live VACV as well as with individual VACV proteins (32) and for passive immunization studies with antisera prepared against VACV and VACV proteins (31); and the intranasal route is believed to be the major avenue for transmission of variola virus. The two anti-B5 chimpanzee/human mAbs, 8AH8AL and 8AH7AL, and a rat anti-B5 mAb, 19C2 (22), were compared for their *in vivo* protective activity. The control mice lost weight continuously starting at day 5 after challenge with 10^5 plaque-forming units (pfu) of WR, and two of the five mice were killed because they reached 70% of starting weight (Fig. 4*a*). In contrast, the mice that were injected with mAbs 8AH8AL or 8AH7AL did not lose weight after the identical challenge with 10^5 pfu of WR, indicating that full protection was achieved. Although the rat mAb 19C2 also protected mice compared with the control mice, substantial weight loss was observed. The two chimpanzee/human mAbs provided significantly better protection than that provided by the rat mAb ($P < 0.0001$ on day 8). The difference in weight loss between the no-antibody control group and each of the immunized groups was also highly significant on day 8 ($P < 0.0001$).

Because there was no difference in protective efficiency between 8AH8AL and 8AH7AL, only the 8AH8AL mAb was used in determining the minimum effective dose of anti-B5. The half-life of the mAb was found to be 6.4 days in mice. Groups of mice were given decreasing doses of 8AH8AL (90, 45, and 22.5 μg per mouse), and a single 5-mg dose of human VIG (2.5 times the recommended human dose on a weight basis) was used for comparison. All five control mice died or were killed when their weight fell to 70% of starting weight (Fig. 4*b*). In contrast, all of the mice injected with 8AH8AL, even at the lowest dose, or with VIG were protected from death after WR challenge. Protection against disease, as measured by the degree of weight loss, however, was dose-dependent for 8AH8AL. The difference in weight loss between mice immunized with 8AH8AL and unimmunized control mice was highly significant on day 7 ($P < 0.0001$ for 90 and 45 μg; $P = 0.0005$ for 22.5 μg). Five milligrams of VIG reduced weight loss after challenge ($P = 0.003$ on day 7). The difference in weight loss between mice receiving 5 mg of VIG and those receiving 45 μg or 90 μg of 8AH8AL was highly significant on day 8 ($P < 0.0001$). No statistically significant difference was found between 5 mg of VIG and 22.5 μg of 8AH8AL.

The therapeutic value of 8AH8AL was assessed by administration of the mAb 2 days after challenge with VACV (Fig. 4*c*). A single 90-μg dose of 8AH8AL administered 48 h after infection protected the mice ($P < 0.0001$ on day 7, versus unimmunized controls), and they experienced only slight weight loss, followed by rapid recovery. In contrast, a single 5-mg dose of VIG administered 48 h after infection afforded much less protection ($P = 0.057$ on day 7, versus unimmunized controls), and two of the five mice were killed because their weight loss reached 30%. The difference in weight loss between the mice receiving the mAb and those receiving VIG was highly significant on day 7 ($P < 0.0001$), indicating that the mAb was more therapeutic than the VIG.

Convalescent sera collected 22 days after challenge from the mice described above (Fig. 4 *b* and *c*) contained negligible amounts of the injected mAbs and were assayed for induced murine antibodies to two EV-associated proteins, B5 and A33, and two MV-associated proteins, L1 and A27, by ELISA. The sera were also assayed for neutralizing antibodies to the MV form of VACV. Only challenged mice that had received antibodies (mAb or VIG) were included because all of the nonimmunized challenged mice had been killed because of weight loss. Sera from mice not challenged with VACV served as negative controls.

Mice that had received VIG either before or after challenge mounted a significant antibody response against all of the proteins tested (Fig. 5 *a–d*), indicating that viral replication and production of VACV had occurred. In contrast, mice that had received the chimpanzee-derived mAb did not mount a significant immune response to either of the two EV membrane proteins; however, they did demonstrate an immune response to both MV membrane-associated proteins, which varied according to the dose and time of mAb administered, suggesting that higher doses of the mAb inhibited virus replication better than lower doses. This dose-dependent pattern of response by the challenged mice was reflected also in their neutralizing antibody response to MV (Fig. 5*e*).

Discussion

Several studies have suggested that antibodies are sufficient to protect against orthopoxvirus infections in mice and monkeys (23, 33, 34). Here we demonstrate that chimpanzee mAbs against VACV B5 protein (an EV-specific protein) alone are sufficient not only to protect mice from lethal challenge with virulent VACV, but also to confer therapeutic protection of mice when administered 2 days after infection. The result is consistent with the previous finding that neutralizing antibodies against EV play a critical role in protective immunity (35, 36).

Our anti-B5 mAbs exhibited much higher protective efficacy than did a rat anti-B5 mAb or human VIG. Competition ELISA showed that chimpanzee/human and rat mAbs did not compete with each other for binding to B5 (data not shown), suggesting that they recognize different epitopes. In addition, the chimpanzee/human mAbs had higher binding affinity than the rat mAb. Noteworthy is that the chimpanzee/human mAbs had a 25-fold slower off-rate than the rat mAb. The difference in binding sites and affinities between the chimpanzee and rat mAbs may contribute to their different protective efficacies. The likely reason that human VIG is inferior to the chimpanzee/human mAbs in animal studies is that the concentration of protective antibodies in VIG is low. Indeed, based on ELISA, we found that 5 mg of VIG contained the equivalent of <10 μg of mAb to B5.

The mAbs to B5 inhibited VACV spread in tissue culture cells, and their effect *in vivo* could have a related explanation. We measured mouse antibodies to two EV membrane proteins (B5 and A33) and to two MV membrane-associated proteins (L1 and A27) as a measure of virus replication. Animals passively immunized with VIG (5 mg) raised antibodies to all four proteins, indicating significant virus replication, which was consistent with the considerable weight loss of these animals. In contrast, antibodies to the VACV proteins were much lower in animals that received the highest amount of mAb (90 μg) and exhibited minimal weight loss. More intriguing were the results obtained with animals receiving 22.5 or 45 μg of mAb. These animals also did not make a response to either of the EV membrane proteins, but they did make a dose-dependent response to the MV membrane-associated proteins. There are several possible explanations for this dichotomy. The simplest is that EV membrane proteins are less immunogenic than MV proteins and that higher amounts of virus replication are needed for a response. However, the protection achieved with the low-dose mAb and VIG was not statistically different. An alternative explanation for the difference in antibody response to EV-specific proteins is that the B5 mAb aggregated progeny EV on the infected cell surface and prevented the induction of antibodies to EV membrane proteins specifically. Indeed, agglutination of prog-

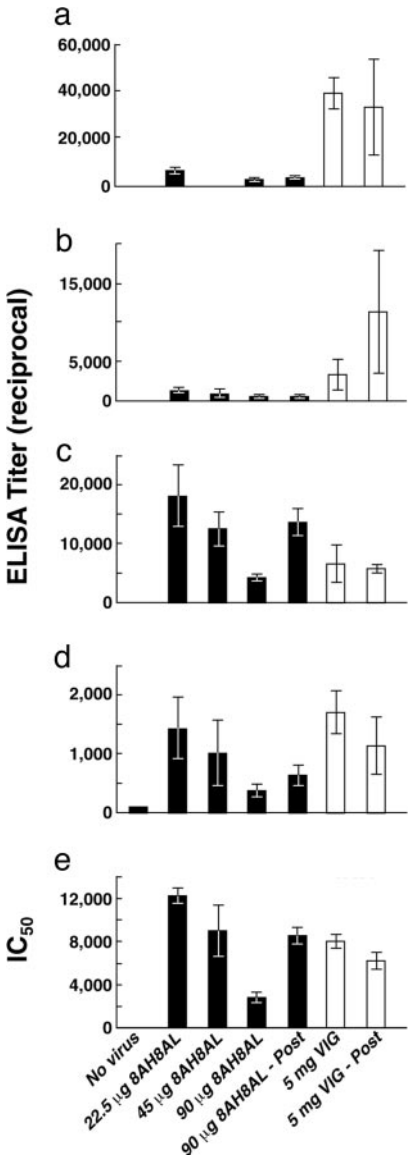

Fig. 5. Antibody responses elicited by challenge with the WR strain of VACV. Mice were bled 22 days after challenge with WR. Individual sera were assayed for binding to EV-associated proteins B5 (*a*) and A33 (*b*) and MV-associated proteins L1 (*c*) and A27 (*d*). The sera were also assayed for neutralizing antibodies to MV (e). IC_{50}, the reciprocal serum dilution that can neutralize 50% of virus. Reciprocal endpoint binding titers were determined by ELISA by using anti-mouse-HRP. Filled and open bars represent animals immunized with 8AH8AL and VIG, respectively. Those groups that received postexposure immunization are indicated by ''Post.''

eny EV on the surface of infected cells has been suggested as the mechanism by which EV antibodies prevent the formation of comet plaques (19).

The use of anti-B5 mAbs in treatment of smallpox vaccine-associated complications would overcome the limitations posed by VIG, such as a low titer of neutralizing activity, variability, and risk of transmission of infectious agents. It is especially important that anti-B5 mAbs crossreacted with variola virus B5 and neutralized variola virus *in vitro*. Amino acid sequence comparison of B5 at residues 20–130 (a neutralization epitope recognized by the anti-B5 mAb) from vaccinia, variola, and monkeypox viruses revealed that there are 10 amino acid differences between vaccinia and variola viruses but only 4 amino acid differences between vaccinia and monkeypox viruses, and 3 of these are the same as in variola virus. Therefore, it is reasonable to assume that anti-B5 mAb would neutralize monkeypox virus also because it can neutralize variola virus. It is conceivable that an anti-B5 mAb alone or in conjunction with other mAbs could be used directly in treatment of bioterrorist-associated smallpox or in case of a monkeypox outbreak (37).

Our anti-B5 mAb recognized a conformational epitope that is located between residues 20 and 130. Previously, two major neutralizing epitopes in B5 had been identified by testing a panel of 26 mouse anti-B5 mAbs; one epitope is localized to the SCR1–SCR2 border, and the other is located in the stalk region (20). The neutralization epitope recognized by the chimpanzee/human mAb may be different from those previously reported (20). However, it is not possible to make a direct comparison because of the different mapping methods used. Our method is based on differential binding of the mAb to a series of N- and C-terminally deleted peptides, and the smallest peptide that still reacted strongly with the mAb was considered to be a binding site, whereas the other method is based on differential binding of a mAb to a series of synthetic, linear, overlapping peptides (20).

In summary, we have generated from the bone marrow of two immunized chimpanzees human-like mAbs that neutralize the extracellular form of VACV as well as that of variola virus. The mAbs protect mice from lethal challenge with virulent VACV and are therapeutic when administered 2 days after exposure. These mAbs provide the first alternative to VIG for treatment of complications of smallpox vaccination and a new approach to the prevention and treatment of smallpox.

Methods

Reagents. Recombinant truncated B5 protein (275t) consisting of amino acids 20–275 was produced in a baculovirus expression system (20) and was used as a panning antigen for selection of B5-reactive phage. Restriction and other enzymes were from New England Biolabs. Oligonucleotides were synthesized by Invitrogen. Anti-His HRP conjugate, anti-human Fab HRP conjugate, and anti-human Fab agarose were purchased from Sigma. VACV WR (ATCC VR-1354), IHD-J (from S. Dales, The Rockefeller University, New York), and VV-NP-siinfekl-EGFP were grown in HeLaS3 cells (ATCC CCL-2.2), purified, and titered in BS-C-1 cells as described in ref. 38. A rat anti-B5 mAb, from hybridoma 19C2 (22), was purified from ascitic fluid (Taconic Biotechnology, Germantown, NY). VIG (Cangene) was obtained from the Centers for Disease Control (C. Allen, Drug Service, Atlanta).

Animals. Chimpanzees 3863 and 3915 were immunized twice ≈19 years apart (initially at Bioqual, Rockville, MD, and subsequently at the University of Texas M. D. Anderson Cancer Center) with VACV WR (39). Bone marrow was aspirated from the iliac crests of these animals 11 weeks after the second immunization. Mice were purchased from Taconic Biotechnology. All animal experiments were performed under protocols approved by the respective institutions as well as by the National Institute of Allergy and Infectious Diseases Animal Care and Use Committee.

Library Construction and Selection. Fab-encoding gene fragments were amplified from the cDNA of chimpanzee bone marrow-derived lymphocytes and cloned into pComb3H vector (40, 41). The phage library was panned against B5 protein, and specific phage clones were selected as described in ref. 42. The details of library construction and selection are provided in *Supporting Text*.

Sequence Analysis. The genes encoding the variable region of the heavy (VH) and light (VL) chains of B5-specific clones were sequenced, and their corresponding amino acid sequences were aligned. The presumed family usage and germ-line origin were established for each VH and VL gene by search of V-Base (26).

Expression and Purification of Fab and IgG. The phagemid containing λ light chain and γ1 heavy chain was cleaved with NheI and SpeI and recircularized after removal of the phage gene III DNA fragment from the vector to encode soluble Fab. Bacteria containing circularized DNA without phage gene III were cultured in 2× YT medium containing 2% glucose, 100 μg/ml ampicillin, and 15 μg/ml tetracycline at 30°C until the OD_{600} reached 0.5–1. The culture was diluted 5-fold in 2× YT medium without glucose and containing 0.2 mM isopropyl β-D-thiogalactoside, and culture was continued at 27°C for 20 h for expression of soluble Fab. Because the Fab was tagged at the C terminus with $(His)_6$, the expressed proteins were readily affinity-purified on a nickel-charged column.

The conversion of Fab to full-length IgG was achieved by digestion of γ1 Fd with XhoI and ApaI and cloning it into pCDHC68B vector (43), which contains the human heavy chain constant region; the λ-chain was cloned into pCNHLCVector3 (43) at XbaI and SacI sites. For full-length IgG expression and purification, plasmids containing heavy chain and light chain were cotransfected into 293T cells for transient expression. The IgG was purified by affinity chromatography with anti-human Fc agarose (Sigma).

The purity of the Fab and IgG was determined by SDS/PAGE, and the protein concentration was determined by bicinchoninic acid assay (Pierce) and spectrophotometer measurement at OD_{280}.

ELISA. B5 (275t) and nonrelated proteins (BSA, cytochrome *c*, thyroglobulin, lysozyme, and phosphorylase b) were coated in a 96-well plate by placing 100 μl containing 1–5 μg/ml protein in 1× PBS (pH 7.4) in each well and incubating the plate at room temperature overnight. Serial dilutions of soluble Fab, IgG, or phage were added to the wells, and plates were incubated for 2 h at room temperature. The plates were washed, and the secondary antibody conjugate (anti-His-HRP, anti-human Fab-HRP, or anti-M13-HRP) was added and incubated for 1 h at room temperature. The plates were washed, and the color was developed by adding tetramethylbenzidine (Sigma). The plates were read at OD_{450} in an ELISA plate reader.

Affinity Measurement. Surface plasmon resonance biosensing experiments were conducted with a Biacore 3000 instrument (Biacore, Piscataway, NJ) by using short carboxy-methylated dextran sensor surfaces (CM3, Biacore) and standard amine coupling as described in detail in ref. 44. The procedure is described in *Supporting Text*.

Epitope Mapping. The epitope recognized by anti-B5 8AH8AL was mapped by Western blot. B5 peptides corresponding to amino acids 20–275, 20–160, 20–130, 20–100, 33–275, 56–275, and 71–275 were synthesized in *Escherichia coli* as described in ref. 45. The analysis is described in *Supporting Text*.

Comet Reduction Assay for VACV. Monolayers of BS-C-1 cells in six-well cell culture plates were infected with the IHD-J strain of VACV, which releases more EV than the WR strain, at 50–100 pfu per well in MEM containing 2.5% FBS (MEM-2.5). After incuba-

tion for 2 h at 37°C, the medium was aspirated, and cells were washed twice and overlaid with MEM-2.5 containing the antibodies to be tested. The plates were then placed in a CO_2 incubator for 36 h. Comets were visualized by staining the monolayers with a solution of 0.1% crystal violet in 20% ethanol. Each mAb was tested at several concentrations (5–30 μg per well). Rabbit polyclonal hyperimmune serum was used as a positive control.

Comet Reduction Assay for Variola Virus. The experiment was carried out in a BSL-4 smallpox laboratory at the Centers for Disease Control. Monolayers of BS-C-40 cells in six-well cell culture plates were infected with the Solaimen strain of variola virus at 50 pfu per well in RPMI medium containing 2% FBS. After 1 h, the medium was aspirated, and cells were washed twice and overlaid with RPMI medium containing antibody at different concentrations. Each treatment was duplicated. The plates were then incubated at a fixed angle in a CO_2 incubator for 4 days at 35.5°C. Cells were fixed and reacted with polyclonal rabbit anti-variola antibody (46). After incubation with goat anti-rabbit–HRP conjugate, comets were visualized by addition of TrueBlue peroxidase substrate (Kirkegaard & Perry Laboratories).

Passive Immunization and Challenge with VACV Strain WR. Groups of 7-week-old female BALB/c mice (Taconic Biotechnology) were inoculated i.p. with antibody diluted in PBS. Nonimmunized controls were injected with the same volume of PBS. Either 24 h after or 48 h before immunization, mice were challenged intranasally with 10^5 pfu of VACV WR as described in ref. 32. Mice were weighed daily for 16 days and killed if their weight diminished to 70% of the initial weight, in accordance with National Institute of Allergy and Infectious Diseases Animal Care and Use protocols. Mice were bled 24 h after passive immunization to monitor administered antibody levels and on day 22 to measure development of antibodies to the challenge virus.

Evaluation of Murine Convalescent Antibody Response After Challenge. Serum samples taken from mice 22 days after challenge with VACV (see above) were analyzed for induction of mouse antibodies to recombinant proteins B5, A33, L1 (47), and A27 and neutralizing antibodies against MV. The recombinant proteins were used to coat 96-well plates as described in ref. 32, and 2-fold serial dilutions of sera were added to the plates. The bound mouse antibodies were detected by anti-mouse IgG(γ)-peroxidase (Roche, Indianapolis). The substrate 3,3′,5,5′-tetramethylbenzidine (BM Blue, POD, Roche) was used, and endpoint titers were calculated as the dilution with absorbance (A_{370} and A_{492}) values two standard deviations above that measured in wells without antibodies. The IC_{50} values for neutralization of the MV form of VACV were determined by flow cytometry by using the reporter virus VV-NP-siinfekl-EGFP as described in ref. 48.

Statistical Analysis. Statistical differences in weight loss between groups of mice were assessed by ANOVA with STATVIEW software (SAS Institute, Cary, NC).

We thank Victoria A. Olson and Kevin Karem for help with comet reduction assay of variola virus. We are grateful to Drs. J. C. Whitbeck, L. Aldaz-Carroll, and Huan Lou (University of Pennsylvania, Philadelphia) for providing us with the vaccine virus proteins A27, L1, B5, and A33. This research was supported by the Intramural Research Program of the National Institutes of Health, National Institute of Allergy and Infectious Diseases. G.C. and R.J.E. were funded by grants from the National Institutes of Health, by National Institute of Allergy and Infectious Diseases Mid-Atlantic Regional Center of Excellence Grant U54 AI057168, and by a University of Pennsylvania Health Research Faculty Development Block Grant.

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